Supporting Information

Light-induced ATP driven self-assembly of actin heavy-meromyosin in a proteo-tubularsomes as a step toward an artificial cell

Satarupa Dhir,[a] Sumalee Salahub,[a] Anu Stella Mathews,[a] Surjith Kumar Kumaran,[a] Anu Carlo Montemagno,[b] Sinoj Abraham *[a,c]

[a] Ingenuity Lab, Chemical and Materials Engineering Department, University of Alberta, Edmonton, Alberta, T6G 2M9, Canada
[b] Southern Illinois University, Carbondale, Illinois 62901, USA
[c] Mechanical Engineering Department, University of Alberta, Edmonton, Alberta T6G 1H9, Canada
*** Corresponding author e-mail sinoj@ualberta.ca.**

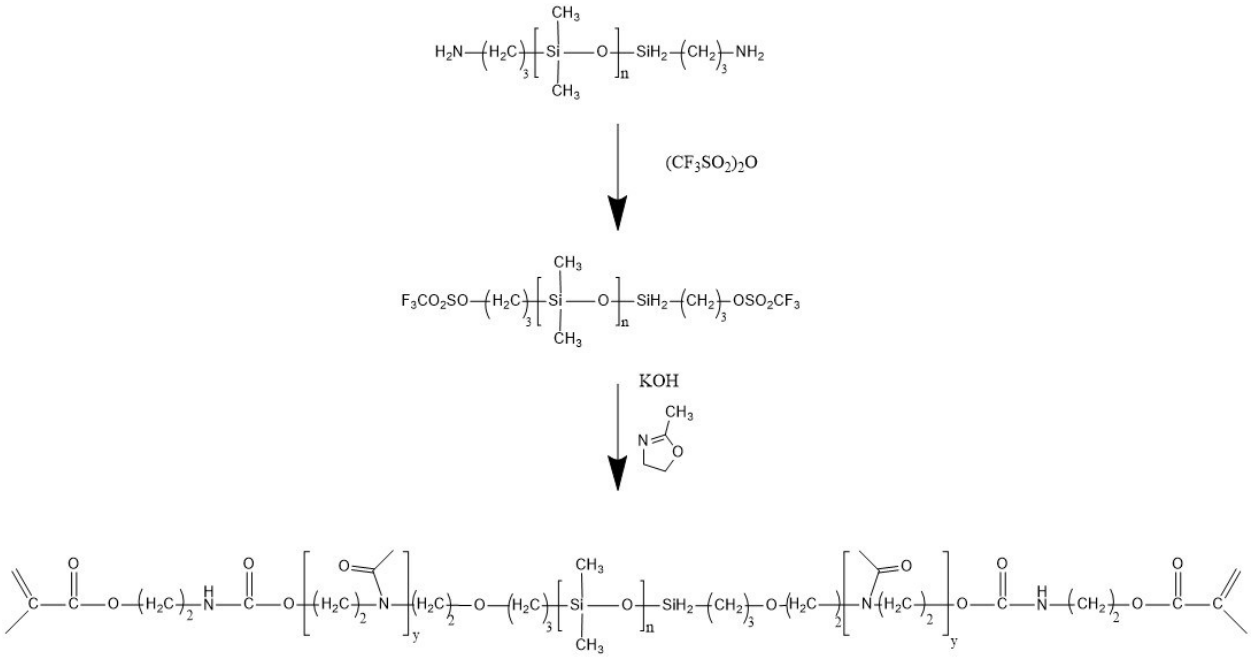


Fig S1: Schematic illustration of PMOXA-PDMS-PMOXA triblock copolymer synthesis by catalytic ring opening polymerization.

Materials

Propargyl alcohol (Aldrich, 99%), p-Toluenesulfonyl chloride(Sigma Aldrich, ≥99%), 4-(dimethylamino)pyridine(Aldrich, ≥99%), sodium hydroxide(Fisher Chemical), sodium sulfate anhydrous(Sigma Aldrich, ACS reagent, ≥99%), potassium carbonate anhydrous(Fisher Chemical), sodium azide(Sigma Aldrich, 98%), Poly(dimethylsiloxane) bis(hydroxyalkyl) terminated (Aldrich, average M_n ~5,600), copper(II) sulfate (Sigma Aldrich, 99%), (+)- sodium L-ascorbate (Aldrich, 99%), triethylamine (Sigma Aldrich), EDTA (Sigma Aldrich), chloroform(

Fisher Chemical), Tetrahydrofuran(Alfa Aesar, HPLC grade), Ethyl acetate anhydrous (Sigma Aldrich, 99.8%) N,N-Dimethylformamide anhydrous(Sigma Aldrich, 99.8%), Dichloromethane anhydrous(Sigma Aldrich, ≥99.8%), 2% uranyl acetate, N,N-Dicyclohexylcarbodiimide (Aldrich, 99%), Tris(hydroxymethyl)aminomethane (Sigma Aldrich, ≥ 99.8%), Calcium chloride anhydrous (Sigma Aldrich, ≥ 93%), Adenosine 5'-triphosphate disodium salt(Sigma, ≥99% (HPLC)), Adenosine 5'-diphosphate sodium salt (Sigma, ≥ 95%), Disodium hydrogen phosphate (Alfa Aesar), Phospho(enol)pyruvic acid monopotassium salt(Aldrich, 99%), Pyruvate Kinase from rabbit muscle(Sigma), Imidazole(Sigma, ≥ 99%), Potassium chloride(Sigma Aldrich, ≥99%), Magnesium chloride anhydrous (Sigma, ≥98%), EGTA (Sigma, ≥ 98%), DL-Dithiothreitol (Sigma Aldrich, ≥ 99%), Methyl cellulose (Sigma, 1500 cp), Valinomycin (Sigma, ≥ 98%), n-Octyl-β-D-Glucopyranoside (Anatrace), Sodium chloride(Sigma Aldrich, ≥ 99.5%), Sodium bicarbonate(Sigma), Zinc chloride(Aldrich, 99%), Rhodamine Phalloidin (molecular probes), Nile Red (molecular probes), Adenosine 5'-triphosphate (ATP) Bioluminescent Assay Kit (FLAA Sigma). 2-ethyl-2-oxazoline (Aldrich, 98%) is purified by distillation and stored in clean flask under Argon atmosphere. Actin protein (> 99% pure from rabbit skeletal muscle) and Heavy Meromyosin Protein (HMM fragment) was purchased from Cytoskeleton Inc.

Purification of bR and F_0F_1 ATPase

Bacteriorhodopsin was expressed *Halobacterium halobium*(S9), a strain of H. halobium which over expresses bR. Bacterial culture was grown as previously described (1). Cells were harvested by centrifugation at 10,000 rcf for 20 minutes using a JLA-9.1000 rotor. Cells which are not immediately lysis for protein purification, were stored at -20 °C. Purification of bR was completed by first isolation of purple membrane (cell membrane fragments with a high content of bR) from cell lysis, delipidation, solubilization, AIEX chromatography, and desalting. Cell pellets were suspended in 10 mL/g of cell pellet in 4.2 mM $MgSO_4$ containing 0.1 mg/mL DNase I and allowed to stir at room temperature until solution was homogenous. The cell lysis was subject to a two-step centrifugation using a 70 Ti rotor. The first step of centrifugation was 9,800 rcf for 15 minutes followed by 25 minutes at 18,400 rcf. The supernatant was decanted and the purple membrane pellet was dilapidated by resuspension at 2 mg/mL in 10 mM MES buffer, pH 5.0 and 81 mM CHAPS and mixed for 24 hours. The removal of excess lipid, CHAPS and contaminating membrane proteins halorhodpsin and sensory rhodopsins I and II was achieved through DF and concentration by TFF using a 0.1 µm cut off filter (Spectrum Labs) with 5 DVs of 50 mM Tris buffer, pH 7.5. The concentration of protein was analyzed using Direct Detect and solubilization was achieved by dilution to 1 mg/mL protein and 25 mM Triton X-100 and incubated for 24 hours. The solubilized protein was filtered through 0.2 µm filter and loaded onto a HiTrap Q FF (CV=5mL) at 30% of the columns protein binding capacity at 5 mL/min. The RB for AIEX was 50 mM Tris buffer, pH 7.5 containing 0.03% (w/w) DDM. After sample loading, a 10CV column wash was performed to completely exchange Triton X-100 with DDM. Following the column wash 5CV linear gradient (5%-100%) of EB (50mM MES buffer, pH 6.0, 1M NaCl, 0.03% (w/w) DDM) was applied and elution of bR was monitored by measured the absorbance at 280 nm and 550 nm. Peak fractions were collected at 2 mL and fractions with OD550/OD280 or greater then

50% were pooled. The pooled fractions were desalted by 3x DF into RB with Amicon Ultra-15 spin tubes (3kD MWCO) spun at 4,000 rcf for 30 minutes. The volume of eluent was replaced with cold RB. The protein was concentrated to 4 mg/mL, aliquoted, flash frozen with liquid nitrogen and stored at - 80°C. A typical purification run would yield 13.4 mg bR/L culture with a purity of 98.6%

Thermophilic ATPase was expressed naturally from *Bacillus sp.* (PS3) with no significant changes from a previously report procedure (2). The purification procedure was also similar to the methods reported (2). The production of Pi by the ATP-hydrolysis was measured using PiColorLockTM phosphate detection reagent Kit (Innova Biosciences) following the manufacturer's instruction. ATPase has multiple subunits of various molecular weights. This makes quantify purity by SDS-PAGE gel analysis very difficult and was not completed. Complications also, arise of measuring concentration due to interference in standard protein quantification assays due to interference from detergents. However, typically 1 L of culture would yield 1000 U of specific activity units of ATPase.

Experimental

Polymer synthesis

The block co-polymer was synthesized utilizing cationic ring-opening polymerization of five-membered 2-oxazoline. Monomer 1,3-bis(4-hydroxybutyl)tetra methyldisiloxane was first initiated with one of the Brönsted acid, trifluoromethanesulfonic acid anhydride to form a living polymer chain. The length of each block was adjusted by addition of varying the amount of freshly distilled 2-ethyl-2-oxazoline. The polymerization was finally terminated by addition of 0.5 M KOH in ethanol. For the current study the PDMS block was maintained at 5600 $gmol^{-1}$while the molecular weight of the end group PEOXA was varied from 1088 to 1396 $gmol^{-1}$.

Polymersomes formation

Polymersomes were prepared by popular "film rehydration method". Film rehydration method is organic solvent free technique. This technique involved dissolving the block copolymers in organic solvent like chloroform with subsequent film formation by evaporation of the solvent. The film was further placed under high vacuum overnight to remove any traces of organic solvent. This was followed by addition of Milli-Q water and with continuous stirring for 4 hours to rehydrate the film. The suspension obtained was then extruded through two polycarbonate membrane filters (Whatman nucleopore membrane) with nominal diameter of 1 µm held in an extruder using 200 psi nitrogen gas. The number of times the sample was extruder was maintained at 10 passes to reach a uniform size distribution.

Determination of critical micelle concentration (CMC)

The critical micelle concentrations of the polymer vesicles were determined by molecular probe method. Briefly, 10 mg of the PEOXA-PDMS-PEOXA polymer was dissolved in 5 mL of chloroform followed by removal of the organic solvent using a rotavaporator. The polymer thin film was formed, placed under high vacuum

overnight, followed by rehydration with 10 mL DI water for 3 hours. The CMC was determined using the change in fluorescence of Nile red dye. Since the dye is hydrophobic, it associates with the hydrophobic core i.e. PDMS of the polymer and strongly fluoresces when micelles are formed. Dilutions ranging from 0.001 to 1.00 mg/mL were prepared from the 1 mg/mL tubularsomes stock solution. To each 1mL of the sample, 10 µL of 1 mg/mL Nile red was then added and incubated at room temperature overnight. The samples were then filtered with 0.45 µm syringe filter and fluorescence was measured in 96 well plates using Microplate reader at excitation wavelength of 535 nm and emission wavelength of 612 nm. Fluorescence data along with DLS measurements were then used to assess the critical micelle concentration of PEOXA-PDMS-PEOXA block copolymer.

Formation of actin bundles and application of HMM

Actin protein (> 99% pure from rabbit skeletal muscle) and Heavy Meromyosin Protein (HMM fragment) were purchased from Cytoskeleton Inc. Actin obtained was polymerized to form F-actin in F buffer: 5 mM Tris HCl pH 8, 0.2 mM $CaCl_2$, 0.2 mM ATP. To form HMM fragments, the solution containing 2 mg/mL of HMM, 10 mM phosphophenol pyruvate (PEP), 20 units/ml pyruvate kinase, 25 mM imidazole HCl, pH 7.6, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM DTT was prepared. To produce non-polar bundles of F-actin, 0.3 % (w/v) of 1,500 cP methylcellulose (MC) was added to the solution of HMM fragments. To form F-actin/HMM bundles, the desired concentration of F-Actin and HMM solution was gently mixed and incubated for 20 minutes so that the MC allowed formation of stable bundles of F-actin/HMM. Ratio of F-actin to HMM played an important role, hence the following ratios were studied to determine the final ratio in tubularsomes: 50 µM F-Actin with 3.8 µM HMM, 50 µM F-Actin with 7.5 µM HMM and 200 µM F-Actin with 13 µM HMM. To visualize the F-actin/HMM bundles rhodamine-phalloidin was added to Actin in molar ratio of 1/40 and incubated at 4 ^{o}C for 1 hour. Control experiments were performed without tubularsomes with initiation of fluorescence by addition of 10 mM ATP.

For incorporation of F-actin/HMM into the tubularsomes, the fluorescent labelled F-actin/HMM was added to thin film of polymer and the rehydrated in 25 mM imidazole HCl, pH 7.6, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM DTT such that final molecular ratio of F-Actin and HMM was maintained at 10. The mixture of F-actin/HMM and tubularsomes were stirred at 4 ^{o}C for 4 hours and dialyzed with 4 changes of buffer using MWCO of 10K membrane.

bR-F_1F_0 ATPase in F-actin HMM tubularsomes

The F-actin/HMM tubularsomes were prepared as mentioned above. To 1 mL of the 1 mg/mL tubularsomes F-actin/HMM, 20 mM n-Octyl-β-D-glucoside was added and stirred using a magnetic bar for 1 hour for solubilization. This was followed by addition of bR (2.5 µL, 3.2 mg/mL) and F_1F_0- ATPase (3.6 µL, 0.5 mg/mL) with another 1 hour of stirring at 4 ^{o}C. The protein tubularsomes mixture was put for dialysis at 4 ^{o}C for 2 days with 4 changes of buffer using 10K MWCO.

For bR-F_1F_0 ATPase incorporation in the tubularsomes, the procedure of film formation and protein addition was exact as mentioned above. However, for faster detergent removal we employed bio-beads. The bio-beads were first washed with 100% methanol to remove any residue and then sonicated under vacuum in the desired buffer. Multiple washes with the desired buffer was carried to equilibrate the bio-beads for their use. After protein incorporation, previously equilibrated wet bio-beads in ratio of 80 mg/mL were added for 1 hr, followed by another 80 mg/mL for another hour and 200 mg/mL for the third hour to ensure complete removal of the detergents.

Fluorescence study of F-actin HMM encapsulated tubularsomes and bR-F_1F_0 ATPase F-actin HMM tubularsomes

For Fluorescence reading, 100 µL of the F-actin/HMM encapsulated tubularsomes was incubated with 5 µl of 100 µM α-Hemolysin for 10 minutes and was excited at 530 nm with emission reading at 576 nm. The control experiments had tubularsomes with α-Hemolysin and ATP and no F-actin/HMM. For 100 µL of bR-F_1F_0 ATPase F-actin/HMM tubularsomes, the proteo-tubularsomes were initiated by 12 µL of 0.2 M ADP and 20 µL of 0.1 M Pi after incubation with 5 µL of 20 µM valinomycin under water bath through 100W mercury lamp. Negative control with 50 µM N,N-Dicyclohexylcarbodiimide (DCCD) a classical inhibitor for F_1F_0-ATPase was adopted for control experiments with three protein system.

Characterization

^{1}H NMR spectra was recorded using a VNMRS 600 spectrometer with VNMRJ 2.2C 1-D acquisition and processing. The FT-IR spectra was recorded using FTS 7000 series Digilab with data analyzed at resolution of 4 cm^{-1} by Varian resolution pro 4.0 after 500 scans of each sample. The scanning electron micrograph in TEM mode was acquired on Hitachi S4800 field emission microscope operating at 30 kV and 20 µA. The polymer vesicles were dropped on carbon-coated TEM grids followed by removal of excess using Kim wipes. One drop of staining solution 2% uranyl acetate was placed on the grid for 30 seconds which was also removed by Kim wipes. The TEM grids were placed under vacuum for 1 hour for solvent removal after which they were analyzed under microscope. Dynamic light scattering (DLS) study was carried out on Malvern Nano ZS zetasizer at room temperature with back scattering angle $\vartheta = 173^{o}$.

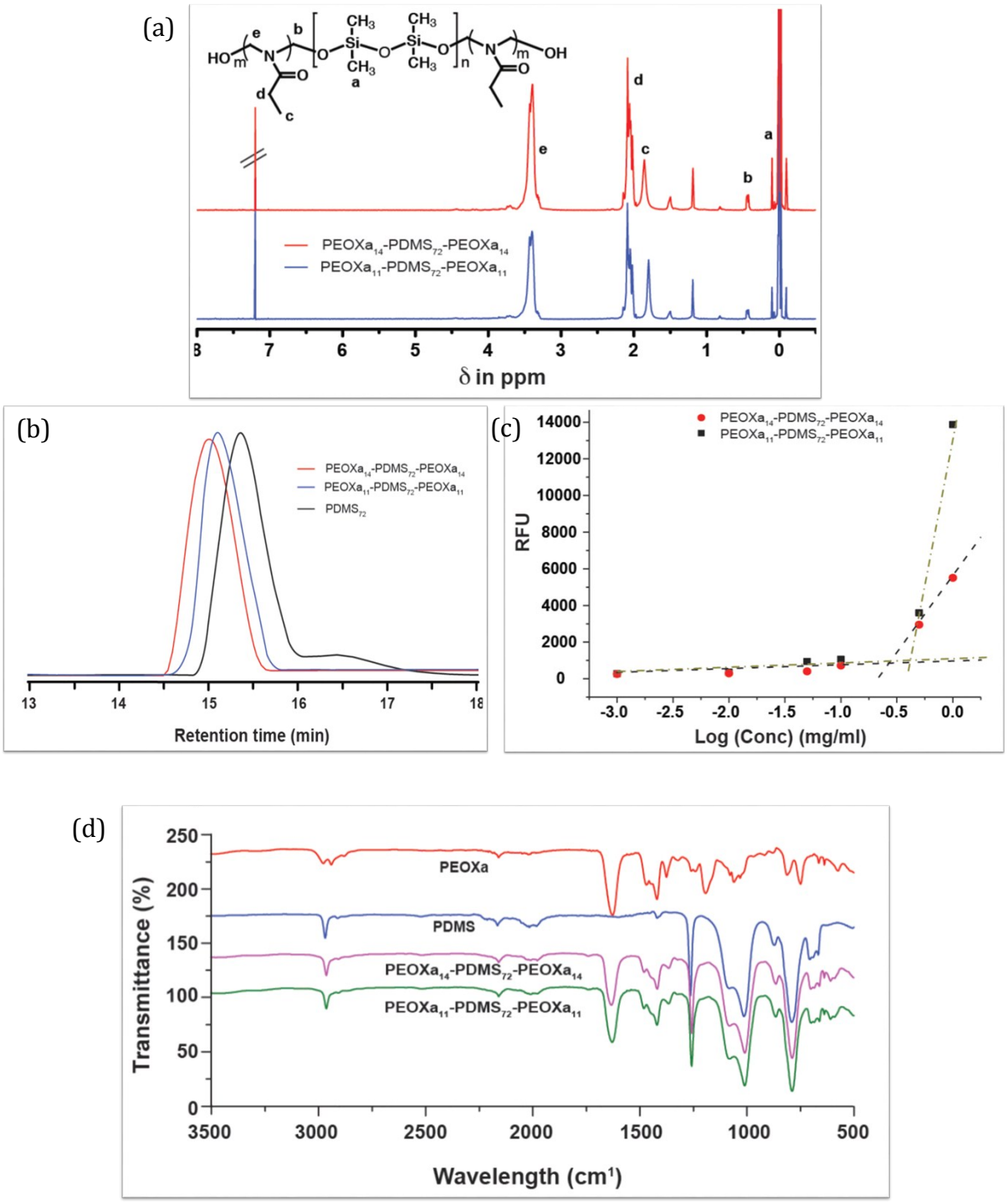
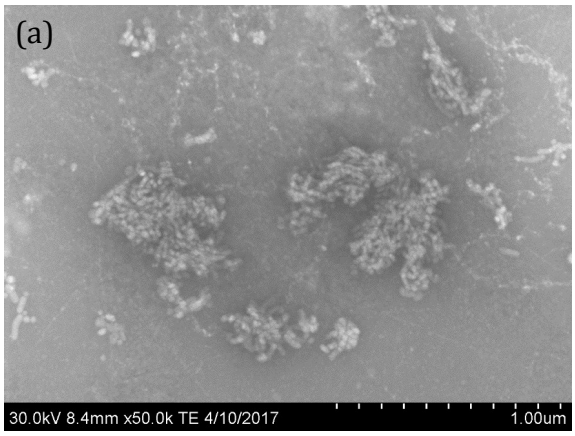


Fig S2: ^{1}H NMR spectrum of PEOXA-b-PDMS-b-PEOX block copolymer (a), Representative of GPC (chloroform) chromatogram of PEOXA-b-PDMS-b-PEOXA, PDMS (b), critical aggregation concentration measurements of PEOXA-b-PDMS-b-PEOXA (c) and FTIR spectra (d).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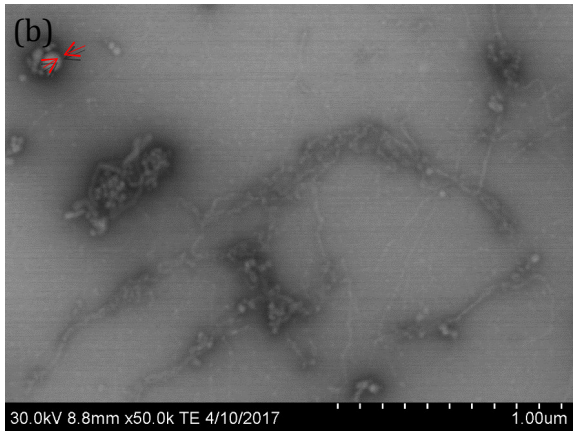
1.00um


(b)
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1.00um

Fig. S3. Scanning electron microscope images of F-actin/HMM self-assembly formed when irradiation with 535 nm light onto bR and F_0F_1 ATPase reconstituted on $PEOXA_{11}$-*b*-$PDMS_{72}$-*b*-$PEOXA_{11}$ (a) and $PEOXA_{14}$-*b*-$PDMS_{72}$-*b*-$PEOXA_{14}$ (b) with 20 μM of ADP/Pi.

Analysis of TEM images using IMAGEJ

Fig S4 illustrated higher resolution image of $PEOXA_{14}$-*b*-$PDMS_{72}$-*b*-$PEOXA_{14}$. The ratio of tubular structures to the spherical ones in $PEOXA_{14}$-*b*-$PDMS_{72}$-*b*-$PEOXA_{14}$ cannot be determined in a quantitative manner but in a qualitative manner. After analyzing the marked areas in the figure, the average diameter of both the tubular and spherical structures are 34 nm with a standard error of 0.72. The ratio of spherical to tubular structures are approximately 27:25, 8:13, 4:5, 5:2 for area A1, A2, A3 and A4 respectively. This ratio is highly variable depending on the aggregation in every TEM grids.

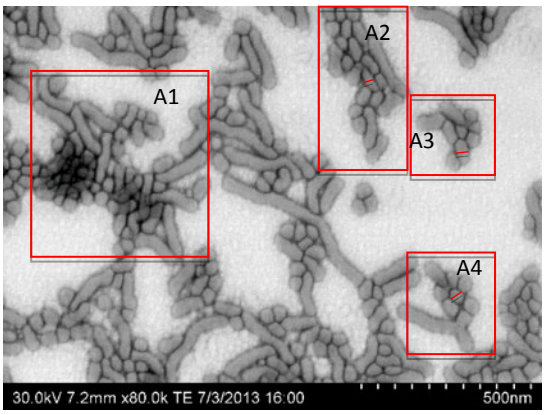

Fig. S4. Scanning electron microscope images of $PEOXA_{14}$-*b*-$PDMS_{72}$-*b*-$PEOXA_{14}$

References

1) Lee, H., et al., Vectorial insertion of bacteriorhodopsin for directed orientation assays in various polymeric biomembranes. *Polymer*, 47, 2935-2941 (2006)
2) Hazard, A. and C. Montemagno, Improved purification for thermophilic F1F0 ATP synthase using n-dodecyl beta-D-maltoside. Archives of Biochemistry and Biophysics, 407,117-124 (2002).